

# Effects of Increased Salinity on Growth, Development and Survival in Early Life Stages of the Green Toad *Bufo variabilis* (Anura: Bufonidae)

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**Abstract** This study examined the effects of increased salinity on growth, development and survival of the Green toad, *Bufo variabilis* during embryonic, hatching and early larval periods. Eggs from a single cohort of *B. variabilis* were subjected to acute and chronic toxicity tests for water salinity ranging from 0.20 to 10 g of salt per liter. Results obtained from this study showed that salinity over 3.70 g/l increased embryonic mortality and reduced percentage of hatching and survival rate of larvae. As larvae tolerated salinity 0.20 to 3.70 g/l with highest survival, but salinity of 10 g/l caused mortality of all individuals within 12 h, 7.70–8.70 (g/l) within 4.5 days, 5.70–6.70 g/l within 10 days and 4.70 (g/l) were tolerated within 14 days. Salinity less than 0.70 g/l did not affect survival and hatching success of the embryos. After a 3-week experiment, size of larvae exposed to salinities over 0.70 g/l was lower compared to larvae reared at lower salinity levels. Meanwhile increases in salinity more than 3.70 g/l caused extension in the hatching period. Larvae reared at salinity of 3.70 to 4.70 g/l had morphological abnormalities, such as distortion of tail.

**Keywords** acute toxicity, *Bufo variabilis*, hatching rate, road salt, salinization, survival

## 1. Introduction

Amphibian populations worldwide are in decline due to a variety of factors including habitat destruction, climate change, diseases, introduction of exotic species, and environmental contamination (Peterson *et al.*, 2002; Stuart *et al.*, 2004). Susceptibility to these factors differs among species and populations (Langhans *et al.*, 2009), and depends, in part, on environmental conditions such as water chemistry (Blaustein and Kiesecker, 2002). Salinity is one of several parameters of water chemistry that influences the survival, development, and fitness of amphibians, and may act as a significant factor in the decline of amphibians (Sparling *et al.*, 2000). Emphasis on the effects of sodium chloride on amphibians has been placed only recently (Karraker and Ruthig, 2009). However, many amphibian species breed in agricultural run off or storm water ponds, which are located along or

close to roads and are expected to come into contact with road de-icing agents that are often applied just before their breeding seasons (Snodgrass *et al.* 2008).

Road de-icers (dry or liquid chemicals able to lower the freezing point of water) are used in temperate and cold regions worldwide when harsh climatic conditions affect road traffic. In most cases, the active agent used in road de-icers is sodium chloride (Ramakrishna and Viraraghavan, 2005). The salts used in de-icers can run off through overland flow, groundwater infiltration and aerosol sprays to reach various wetlands in the washbasin and causes contamination (Marsalek, 2003; Karraker, 2008). De-icing agents, primarily road salt, are applied to roads in 26 states in the United States (Daley *et al.*, 2009) and in a number of European countries (Karraker, 2007; Wijethunga *et al.*, 2016), however, the scale of impacts of road salt on aquatic organisms remains largely understudied (Corsi *et al.*, 2010; Findlay and Kelly, 2011; Petranksa and Francis, 2013; Niyogi *et al.*, 2016). In Iran a mixture of sand and salt is normally used as road de-icer in western and northern part of the country where there is normally a long period of freezing in winter. There are

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Received: 2 November 2017 Accepted: 11 February 2018

reports of associations between road de-icing, salinity in ground water along highway (Reyahi *et al.*, 2011) and also in surface run off (Aghazadeh *et al.*, 2012) in Iran.

Salinity is one of the important environmental factors affecting survival, growth and distribution of many aquatic organisms (Kumlu *et al.*, 2000; Chand *et al.*, 2015). Most amphibians do not adapt well to brackish water because their skin is highly permeable, posing problems for water and ion balance (Duellman and Trueb, 1986). Moderate salinity of 2–9 ppt (parts per thousand) can decrease survival, growth, and development (Chinathamby *et al.*, 2006; Sanzo and Hecnar, 2006), and cause morphological abnormalities, such as distortion of tail, abdominal edema and emaciated appearance together with reduction in body water content (Chinathamby *et al.*, 2006; Karraker, 2007). Despite the evidences provided for some populations of amphibian showing that they can locally adapt to saline environments, evidence is emerging that this may not always be possible (Brady, 2013). Several reports of populations of various species such as *Rana temporaria*, *Buergeria japonica*, *Kaloula pulchra*, *Microhyla ornata*, and *Bufo bufo* have further concluded that salinity causes negative effects on survival, growth, development, metamorphosis and behavior of tadpoles (Gordon *et al.*, 1961; Ackrill *et al.*, 1969; Ferreira and Jesus, 1973; Viertel, 1999; Haramura, 2007; Karraker *et al.*, 2010). In contrast, other species, such as *Fejervarya cancrivora*, *F. limnocharis*, *Bufo balearicus*, *Epidalea calamita*, *Litoria aurea* and *Hoplobatrachus tigerinus* can tolerate higher salinity by maintaining high level of urea in the blood (e. g., *F. cancrivora*) or through gradual acclimation to increasing salinity in laboratory experiments (Christy and Dickman, 2002; Gomez-Mestre and Tejedo, 2003; Wu and Kam, 2009).

Most studies on the effects of salt on amphibians have focused on the adult or larval stage, ignoring the eggs, despite some evidence suggesting that embryos and tadpoles may in fact be the most susceptible life-history stage to salt (Karraker and Ruthig, 2009; Nakkrasae *et al.*, 2016). Adult amphibians rely on integumentary system to retain body fluids through ionic exchange and the ability to hyper synthesize and retain urea to increase body osmolarity (Shoemaker and Nagy, 1977; Balinsky, 1981; Katz, 1989). The green toad, *Bufo variabilis* (Pallas, 1769), is a common toad of Iran and has a wide distribution in most provinces. This toad is rarely found in central and eastern areas of Iran (Masshaii *et al.*, 2008). Whether salinity change interferes with hatching, survival, growth and development of *B. variabilis* tadpoles is unknown. Therefore, main purposes of this

study are to examine the influence of salinity on the (1) growth, (2) development and (3) survival of embryos and larvae of *B. variabilis*.

## 2. Materials and Methods

Eggs (embryos within their jelly capsule, Gosner Stage 10–11) from a single cohort of *B. variabilis* were collected on May 2016 in Sarable (34°32' N, 47°01' E), Kermanshah Province, Iran. All eggs of the same trial were at the same stage. Eggs were cultured at various salinities, 0.20, 0.70, 1.70, 2.70, 3.70, 4.70, 5.70, 6.70, 7.70, 8.70 and 10 g/l. The salinity we used in this study has been determined based on the expected toleration of a freshwater amphibians reported in the scientific literature ranging from tap water (0.20 g/l) to hyper saline water (10 g/l). Freshwater is generally defined as water in which salinity is less than 3 g/l and sea water as 35 g/l (Nielsen *et al.*, 2003). Each treatment was replicated 3 times for a total of 33 containers (14 cm height and 14 cm diameter), each filled with 2l of dechlorinated tap water with salinity of 0.20 g/l. All containers had 20 eggs, Gosner Stage 14–15. In this study developmental stages are followed as defined by Gosner (1960): embryo <25, hatchling 17–20, larvae >25. The experiment was conducted on a 12h dark: 12h light photoperiod at approximately 18°C. Light was supplied by 18 metal halide lamps hanged over laboratory benches to provide a broad spectrum of photosynthetically available irradiant. After hatching larvae were fed with raw spinach: 1 g four times per week for every container. We monitored experimental containers twice a day and removed the bodies of larvae that had died. The larvae were taken out and container were cleaned thoroughly. We did not use any chemical cleaner for this purpose.

We evaluated the impact of salt water on growth of body size by measuring changes in snout to vent length (SVL) during larval period. Regression equations were used to derive the growth rates from the values of length of SVL. Survival was determined as the percent of remaining individuals during embryo and larval period. Photos were taken with a digital camera (SONY, DSC-HX9V, 3.6V) on a tripod at a fixed height (30 cm). The larvae were put in a Petri dish which was located over latticed paper. Immediately after photography the larvae were released into their containers. All pictures were analyzed using Digimizer version 4.6.0 (<http://digimizer.findmysoft.com/>). We measured the snout to vent length (SVL: mm). SVLs were calculated by drawing a line from the tip of the snout to the tip of the vent. Measurements were performed at days 1, 6, 12, 18 and 24. When

experiment was completed, the surviving larvae were returned to the pond where they were collected. One-way analysis of variance (ANOVA) was used to examine the effects of salinity on the rate of hatching and on growth, development and survival during embryonic, and larval period. All data are expressed as mean  $\pm$  SD (standard deviation). The statistical program package SPSS (v. 16) was used for all analyses.

### 3. Results

In this study, salinity was found to markedly affect the rate of hatching among treatments (ANOVA,  $P \leq 0.001$ ). Also, increase in water salinity extended hatching period. Eggs in salinity of 0.20 g/l to 3.70 g/l were hatched after 72 hours while eggs in salinity of 4.70 g/l to 5.70 g/l were hatched after 79.92 hours days. More delay in hatching was found for eggs in salinity 6.70 to 8.70 g/l, were hatched after 96 hours (Table 1). At salinity level of 10 g/l, all eggs shrank and died before hatching. Most unhatched eggs died as indicated by signs of opaqueness and shrinkage, while few eggs were alive but did not hatch. After being exposed to saline water for 24 h post hatching, there was a significant effect of salinity on survival (ANOVA,  $P \leq 0.001$ ) of larvae. Approximately, 83.33% of hatched larvae in 0.20 g/l survived for 24 h. Hatched larvae in salinities of 0.70, 1.70, 2.70, 3.70, 4.70, 5.70 and 6.70 g/l showed a survival rate of 71.66%, 78.33%, 80.66%, 78.66%, 75.33%, 70% and 66.33%, respectively, 24 h after hatching (Figure 1A). The percentage of survival of larvae to the end of the experiment (Gosner stage 30) was 80.00%, 66.66%, 76.66%, 65%, 30% and 0.00% for the 0.20, 0.70, 1.70, 2.70, 3.70 and 4.70 to 10 g/l treatments, respectively

(Table 1, Figure 1). At salinities over 8.70 g/l unhatched and dead embryos appeared with signs of shrinkage and distorted appearance.

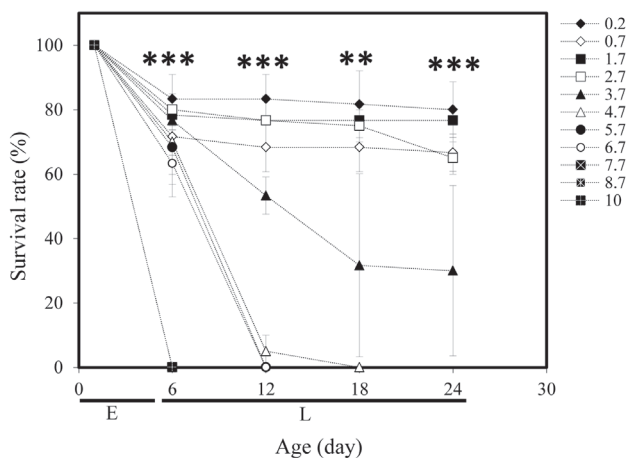
Egg diameter of *B. variabilis* was on average 1.51 mm  $\pm$  0.01, (Gosner stage 10–11) and jelly capsules diameter was 3.76 mm  $\pm$  0.12. Figure 2 and Table 1 demonstrates the average and standard deviation of the (SVL) during larval period of *B. variabilis* from 6 to 24 days. Growth of *B. variabilis* showed significant difference in the third week ( $P \leq 0.05$ ), (Figure 1B). Growth rate for SVL of larvae of *B. variabilis* are shown in Table 1. Larvae growth rate of the 0.70 g/l (Linear regression, 0.26 mm/day) was fastest than 0.20 g/l (Linear regression, 0.24 mm/day) and followed by the 1.70 g/l (Linear regression, 0.23 mm/day), 2.70 g/l (Linear regression, 0.22 mm/day) and 3.70 g/l (Linear regression, 0.15 mm/day). Various salinity treatments affected developmental rate in live embryos and larvae but this difference was not significant (ANOVA,  $P \leq 0.07$ ). The Gosner stages for development of the eggs of the same clutch reared at four treatments of various salinity treatments at 6 to 24 days are shown in Table 1.

### 4. Discussions

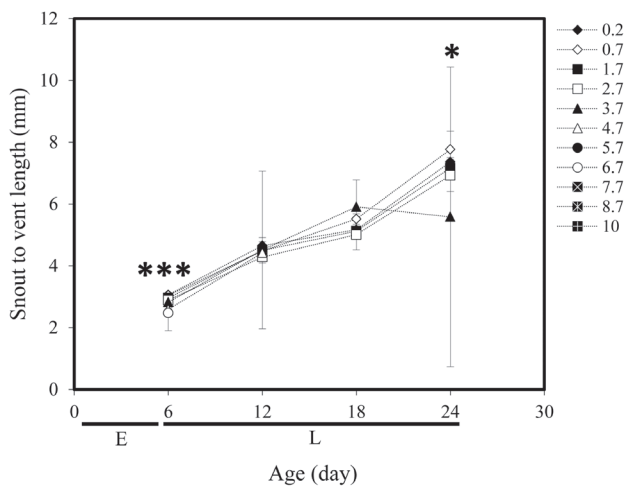
The increase in water salinity in wetland and aquatic ecosystems can result from natural factors such as climate change or sea water intrusion into freshwater wetlands. Various man-made processes such as deforestation, excessive irrigation, salt mining, and road de-icing cause changes in water salinity (Nielsen and Brock, 2009). Increase in salt content in natural aquatic ecosystems under natural or anthropogenic processes is now recognized as a threat to the biological communities

**Table 1** Percentage and time of hatching (Gosner stage 20) and survival rate of *Bufo variabilis* larvae in various salinity treatments for 24 h (Gosner stage 26) and 24 days (Gosner stage 30) after hatching. Snout to vent length (SVL: Mean  $\pm$  SD) was measured at 6 and 24 days. Growth rate (mm/day) of body size (SVL) is determined as daily increase of SVL during 6 and 24 days.

Salinity (g/l)	Hatching time (hours)	% Hatching	% Survival 24-h post hatching	% Survival at 24 Day	SVL (mm) at 6 Day	SVL (mm) at 24 Day	Growth rate (mm/day)
0.2	72	91.66 $\pm$ 2.88	83.33 $\pm$ 7.63	80.00 $\pm$ 8.66	3.06 $\pm$ 0.06	7.36 $\pm$ 0.38	0.24
0.7	72	88.33 $\pm$ 2.88	71.66 $\pm$ 5.77	66.66 $\pm$ 5.77	3.04 $\pm$ 0.07	7.76 $\pm$ 0.58	0.26
1.7	72	86.66 $\pm$ 2.88	78.33 $\pm$ 11.54	76.66 $\pm$ 10.40	2.95 $\pm$ 0.05	7.16 $\pm$ 0.34	0.23
2.7	72	81.66 $\pm$ 5.77	80.66 $\pm$ 5.00	65.00 $\pm$ 5.00	2.89 $\pm$ 0.06	6.92 $\pm$ 0.51	0.22
3.7	72	83.33 $\pm$ 7.63	78.66 $\pm$ 11.54	30.00 $\pm$ 5.30	2.82 $\pm$ 0.04	5.58 $\pm$ 0.45	0.15
4.7	79.92	73.33 $\pm$ 11.54	75.33 $\pm$ 10.44	0	2.58 $\pm$ 0.01	-	-
5.7	79.92	70.00 $\pm$ 5.00	70 $\pm$ 11.54	0	2.47 $\pm$ 0.57	-	-
6.7	96	73.33 $\pm$ 2.88	66.33 $\pm$ 10.40	0	2.47 $\pm$ 0.04	-	-
7.7	96	50.00 $\pm$ 8.66	0	0	-	-	-
8.7	96	33.33 $\pm$ 11.54	0	0	-	-	-
10	0	0	0	0	-	-	-



**Figure 1** Effect of different salinities on survival rate (%) of embryo (E) and larvae (L) of *Bufo variabilis* from 1 to 24 days (age). \*\*\*:  $P \leq 0.001$ ; \*\*:  $P \leq 0.01$ ; \*:  $P \leq 0.05$ .



**Figure 2** Effect of different salinities on snout to vent length (mm) of larvae (L) of *Bufo variabilis* from 1 to 24 days (age). \*\*\*:  $P \leq 0.001$ ; \*\*:  $P \leq 0.01$ ; \*:  $P \leq 0.05$ .

as a whole and represents an environmental stress for many species (Jin *et al.*, 2011). Amphibians with their permeable skin are at risk in hyposaline and hypersaline water, because they gain or lose water across the skin surface at rates that may rapidly be fatal (Wijethunga *et al.*, 2016). A highly permeable skin makes amphibian osmotically sensitive organisms, because their osmoregulation works at a certain range of water salinity (Gomez-Mestre *et al.*, 2004; Haramura, 2007).

Experimental evaluation of the impact of elevated salinity normally showed a considerable reduction in growth, and the rate of metamorphosis. These are also reports of associations between an increase in mortality rate in anuran adults and larvae (Christy and Dickman, 2002). There is a general consensus in the literature that

amphibian embryos are most sensitive to water salinity, followed by larvae, with adults being most tolerant (e. g., Chinathamby *et al.*, 2006; Brand *et al.*, 2010; Petranka and Doyle, 2010; Bernabé *et al.*, 2013; Hopkins *et al.*, 2014; Thirion, 2014). There are also some opposing data and evidence that show the sensitivity can also change with age within a particular life stage (Alexander *et al.*, 2012).

Recent investigations indicated that de-icing salts were associated with reduced survival and increased frequency of malformations in some amphibians e.g. the Spotted Salamander, *Ambystoma maculatum*, and the Wood Frog *Rana sylvatica* (Nakkrasae *et al.*, 2015). A reduction in survival in embryonic *A. maculatum* and *R. sylvatica* was probably caused by physiological constraints imposed on embryos at higher salinities (Nakkrasae *et al.*, 2015). In contrast, survival of embryos and larvae of green frogs (*Lithobates clamitans*), which breed principally in permanent wetlands, was not significantly influenced but frequency of malformations increased with chloride concentration (Karraker, 2007). While a reduction in growth may result from the increased energy expense required for osmoregulation, delayed development may result from a decreased thyroid hormone (TH) level in response to hormonal involvement in osmoregulation (Gomez-Mestre *et al.*, 2004).

Present study covered embryonic, hatching and early larval periods in the course of 20 days after hatching. On this short period of exposure to different water salinity the reaction was slow or limited to retard development of eggs, hatchlings and larvae (Table 1) which may eventually lead to smaller size at metamorphosis. Whether this can result in a reduction in survival rate in adult is not known. There are several studies that show small body size at metamorphosis decreases the ability to tolerate dehydration (Newman and Dunham, 1994) and the likelihood of post-metamorphic survival (Smith, 1987; Berven, 1990; Chelgren *et al.*, 2006). Present study did not cover entire developmental period but previous studies showed that time to metamorphosis of larvae decreased when reared in intermediate salinity treatments compared with freshwater or low salinity treatment. (Chinathamby *et al.*, 2006; Sanzo and Hecnar, 2006; Wu and Kam, 2009).

The Green Toad (*B. variabilis*) of Europe and the Middle East (Gordon, 1962) have been reported from aquatic environments of salinities as high as 2 g/l, in northern Europe (Gislén and Kauri, 1959). Results obtained from current experiment showed that impact of salinity on embryonic mortality, hatching and survival



rate of larvae began at concentration over 3.70 g/l. Although present salinity of freshwater wetland where the eggs have been collected for this experiment is much lower than the water salinity in the laboratory but the interaction of a shortened metamorphosis period and a smaller body size at high salinity with other ecological factors is difficult to anticipate under natural condition. There are now increasing evidences showing that in many regions of the world a net negative impact of the recent climate change is expected to occur in freshwater ecosystems (IPCC 2007). However, the intensity and characteristics of the impact can significantly vary from region to region. In many arid and semi-arid regions such as Iran, trends toward warmer climate and increased precipitation variability are linked to warming streams and rivers (IPCC 2007). Amphibians are well adapted to environmental fluctuations but the novel situation resulting from the combined impact of various environmental factors may cause an irreversible impact on their existence.

**Acknowledgements** We thank the Razi University for the support of this study as an MSc research project. Collection of Green Toad eggs was permitted by the Regional Office of Environment in Kermanshah Province.

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